2009

Evolution and Systematic Value of Leaf Crystal Macropatterns in the Genus Peperomia (Piperaceae)

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Abstract
Leaves of Peperomia species vary in texture, shape, succulence, lamina size, thickness, coloration, and venation. Each leaf contains only calcium oxalate druses in palisade cells, druses in palisade and raphides in spongy parenchyma, or druses in palisade and prisms in spongy parenchyma. Collectively, these variations create distinct crystal macropatterns. Leaves from 45 species were studied to identify their macropatterns and to compare the macropatterns with molecular data. Microscopic data showed two major crystal macropatterns and five variations of them. All but one species displayed either a single druse in most palisade cells forming one or more uniform crystal layers (81.8%, uniform) or a single druse per cell in palisade cells only above veins forming a reticulate pattern (18.2%, reticulate). Most species (77.3%) did not display any crystals in spongy parenchyma; however, some clades showed spongy parenchyma raphide bundles while others had prisms. Several clades displayed more than one macropattern. Based only on crystal macropatterns, data did not demonstrate a specific phylogenetic trend and failed as synapomorphies for most of the clades. However, crystal macropattern evolution in Peperomia is generally characterized by an increasing complexity of the distribution of druses, raphides, and prisms, with few reversals.

Keywords
calcium oxalate crystals, clearings, druses, leaf crystal macropatterns, prisms, raphides

Comments
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Source: *International Journal of Plant Sciences*, Vol. 170, No. 3 (March/April 2009), pp. 343-354
Published by: The University of Chicago Press
Stable URL: http://www.jstor.org/stable/10.1086/596338
Accessed: 14-06-2016 13:24 UTC

Int. J. Plant Sci. 170(3):343–354. 2009.

1058-5893/2009/17003-0007$15.00 DOI: 10.1086/596338

EVOLUTION AND SYSTEMATIC VALUE OF LEAF CRYSTAL MACROPATTERNS IN THE GENUS *PEPEROMIA* (PIPERACEAE)

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Leaves of *Peperomia* species vary in texture, shape, succulence, lamina size, thickness, coloration, and venation. Each leaf contains only calcium oxalate druses in palisade cells, druses in palisade and raphides in spongy parenchyma, or druses in palisade and prisms in spongy parenchyma. Collectively, these variations create distinct crystal macropatterns. Leaves from 45 species were studied to identify their macropatterns and to compare the macropatterns with molecular data. Microscopic data showed two major crystal macropatterns and five variations of them. All but one species displayed either a single druse in most palisade cells forming one or more uniform crystal layers (81.8%, uniform) or a single druse per cell in palisade cells only above veins forming a reticulate pattern (18.2%, reticulate). Most species (77.3%) did not display any crystals in spongy parenchyma; however, some clades showed spongy parenchyma raphide bundles while others had prisms. Several clades displayed more than one macropattern. Based only on crystal macropatterns, data did not demonstrate a specific phylogenetic trend and failed as synapomorphies for most of the clades. However, crystal macropattern evolution in *Peperomia* is generally characterized by an increasing complexity of the distribution of druses, raphides, and prisms, with few reversals.

Keywords: calcium oxalate crystals, clearings, druses, leaf crystal macropatterns, prisms, raphides.

Introduction

Calcium oxalate is an inorganic compound that is produced extracellularly and intracellularly by a variety of animals (Hodgkinson 1977), microorganisms (Khan 1995), and plants (Arnott and Pautard 1970; Franceschi and Horner 1980; Horner and Wagner 1995; Franceschi and Nakata 2005). The calcium is environmentally derived, whereas the oxalate is produced metabolically by at least one of several pathways (Hodgkinson 1977; Franceschi and Horner 1980; Franceschi and Nakata 2005). The functional significance of this inorganic compound continues to be debated, particularly with respect to microorganisms and plants. In animals and humans, it is basically considered a waste product that may cause kidney and bladder stones in the urinary tract and may form in other nonurinary tissues (Hodgkinson 1977). However, microorganisms and plants seem to tolerate calcium oxalate in whichever organ(s) and tissue(s) it forms, and through evolution, microorganisms and plants may have adapted calcium oxalate for use in different structural and physiological ways (Franceschi and Horner 1980; Nakata 2003; Braissant et al. 2004; Franceschi and Nakata 2005).

Calcium oxalate crystals consist of two hydration states (Frey-Wyssling 1981) and appear as several distinct forms or shapes (Franceschi and Horner 1980; Franceschi and Nakata 2005; Hartl et al. 2007). In vascular plants, there are at least five general forms that are recognized: needlelike raphides in bundles, prisms, styloids, crystal sand, and druses (spherical aggregates of crystals). These different forms and variations of them may occur in tissues of different organs, and they are reported to be species specific and under genetic control (Kausch and Horner 1982).

Anthony Van Leeuwenhoek is recognized as the first individual to have identified raphide crystals microscopically in the plant *Arum* (Araceae). Since then, crystals of various morphologies have been described in green algae, ferns, gymnosperms, and angiosperms, the latter group identified in the widely used generalization that about 75% of all flowering plants contain them (Zindler-Frank 1976). The crystals can be seen in freehand, paraffin, and resin sections of organs but are best observed when clearings are made of the different plant organs, especially the leaves. Observation of these latter preparations between crossed polarizers generates vivid, sometimes multicolored, crystal arrays.

Literature about plant crystals has been erratic over many years (Metcalfe and Chalk 1950); however, in the past ~50 yr, there has been renewed interest in their cellular development (Arnott and Pautard 1970; Horner and Whitmoyer 1972; Franceschi and Horner 1980; Webb 1999), their specific structure and location (Metcalfe and Chalk 1950; Horner and Wagner 1980, 1992; Metcalfe 1983), their function (Nakata 2003; Franceschi and Nakata 2005; Kostman et al. 2007; Nakata and McConn 2007), and, most recently, whether they may

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Manuscript received May 2008; revised manuscript received September 2008.

be a useful taxonomic character at the family (Zindler-Frank 1987; Prychid and Rudall 1999; Monje and Baran 2002; Hartl et al. 2007) and subfamily and genus levels (Lersten and Horner 2000, 2004, 2005*a*, 2005*b*, 2006, 2007, 2008; Cervantes-Martinez et al. 2005).

With the advent of molecular systematics, there have been many studies that have utilized molecular data to elucidate relationships among organisms from a viewpoint other than morphology and anatomy. However, crystal macropatterns, the forms and locations of the crystals present in the leaves (macropatterns sensu Lersten and Horner 2000), have never before been combined with molecular data. This study of crystal macropatterns in *Peperomia* species deals with a marriage of these two approaches to determine the extent to which they complement each other.

Detailed morphological, ultrastructural, and anatomical studies have been and continue to be used to find synapomorphies or unique character combinations defining clades in *Peperomia* since the beginning of research in *Peperomia* (Miquel 1843; Dahlstedt 1900; Trelease and Yuncker 1950). However, a recent study (Samain et al. 2009) has shown that many traditionally used characters have been subject to parallel evolution. Hence, the genus *Peperomia* is lacking synapomorphies for infrageneric groups. This is not only an issue in *Peperomia* but also in many species-rich angiosperm clades, such as *Senecio* (Pelser et al. 2002), *Euphorbia* (Bruyns et al. 2006), *Begonia* (Forrest et al. 2005), *Impatiens* (Janssens et al. 2006), and *Pilea* (Monro 2006). Searching for new synapomorphic characters for infrageneric clades, this study tests calcium oxalate crystal macropatterns for their systematic value in a species-rich clade by mapping them on an available and accepted backbone phylogeny of the genus *Peperomia*.

Material and Methods

Species used to study crystal macropatterns were chosen following the sampling of recent molecular and morphological studies in *Peperomia*, where the species represent all currently known infrageneric clades of the genus. Specimens used in this study are mostly identical to those used for the phylogeny of Wanke et al. (2006) and Samain et al. (2009). This relatively limited number of species sampled has proven to be highly representative for the whole genus based on the currently largely extended sampling performed in our ongoing research (H. T. Horner, S. Wanke, and M.-S. Samain, unpublished data). Table 1 shows the 45 investigated species with affiliation to their respective clades according to Samain et al. (2007, 2009). Voucher information can be obtained from table 2 of Wanke et al. (2006). Specimens for this study were obtained from living plants growing at the botanical gardens of Ghent University, Gent, Belgium, and the Technical University, Dresden, Germany; some herbarium specimens (GENT, DR) were also used. In addition, some of the same species were available in two greenhouses and in the Ada Hayden Herbarium (ISC), all at Iowa State University, Ames, and were compared for confirmation of crystal macropatterns. Samples were taken from more than one healthy mature leaf, and at least three samples were observed from each species.

Preparation of Samples

Leaf samples were collected by using a cork borer, by cutting small squares with a clean razor blade, or by using intact whole small leaves. The leaf samples were immersed in 70% ethanol in perforated ice cube trays in baking dishes for easy processing and transferring from one solution to another (Horner and Arnott 1961). They were heated to 60°C in an oven for ~12 h (to remove leaf pigments). Then they were washed several times with deionized water (deiwater) and treated for one to several days with household bleach (5% sodium hypochlorite) to remove all cell cytoplasms. Cell walls and inorganic calcium oxalate crystals were not affected by this treatment. After bleach treatment, samples were thoroughly washed in running tap water for several hours. Following two deiwater rinses, the samples were then dehydrated through increasing concentrations of ethanol (25%, 50%, 70%, 95%, and twice at 100%, 30 min or more per step), and then through 1 : 1 100% ethanol : xylol and into pure xylol, where the samples were rendered clear. The samples were then infiltrated with Permount mounting medium (http://www.fishersci.com), placed on glass slides in Permount, and coverslipped. A lead weight was added to the top of each coverslip to keep the thick specimens as flat as possible during drying. The slides were placed on a warming tray to harden the Permount at the edges of the coverslips. The samples were observed between crossed polarizers using an Olympus BH 10 compound microscope (http://www.olympus-global.com), and the images were calibrated and captured with a Zeiss Axiocam MRc digital camera (http://www.zeiss.de) mounted on the microscope.

Crystal Identifications, Leaf Anatomy, and Druse Diameter Measurements

Mounted samples of the 45 species were observed between crossed polarizers using a light microscope. The form(s) of the crystals and their location(s) were recorded.

Some fresh leaf samples were sliced into 50–70-μm-thick cross sections using a Vibratome 3000 (http://goliath.ecnext.com) to verify leaf anatomy. Even though most of the species were not examined in this way, the general leaf anatomy was consistent with previous reports (Haberlandt 1904; Schürhoff 1908; Franceschi and Horner 1980; F. Foulon, M.-S. Samain, and P. Goetghebeur, unpublished data).

The palisade parenchyma druses in the clearings were visualized between crossed polarizers using a 20× objective. Calibrated captured images of druses were imported into the SIS AnalySIS (http://www.olympus-global.com) program, where they were recalibrated for making diameter measurements. More than 75 druse diameters from five or six leaf regions were made, and the program calculated the statistical data automatically. These data were derived for druses displaying all macropatterns (see "Results"; table 1).

Reconstructing Character Evolution Using Parsimony

DNA isolation, amplification, and sequencing methods are described by Wanke et al. (2006, 2007). Phylogenetic hypotheses were generated with a maximum parsimony approach as a basis for the distribution of morphological characters and the estimation of changes during evolution. Cladistic analysis

Table 1

Clade Affiliation, Crystal Macropatterns, and Palisade Parenchyma Druse Diameters (μm) from 44 *Peperomia* species

Species	Infrageneric clade	Crystal macropattern	Count	Mean diameter (μm)	SD (μm)	Maximum diameter (μm)	Minimum diameter (μm)
P. andina var. *pseudoperuviana*	*Tildenia*	U−/− prisms associated with druses	130	4.27	.56	5.57	3.12
P. argyreia	Currently unnamed	R−/−	60	18.55	2.62	24.66	11.64
P. bicolor	*Micropiper*	U−/−	105	7.86	1.19	10.69	5.52
P. blanda	*Micropiper*	U−/−	136	8.02	1.05	11.28	5.64
P. clusiifolia	*Oxyrhynchum*	U−/−	85	7.83	1.19	11.57	5.60
P. cotyledon	*Panicularia*	R−/− few areole druses	191	12.21	1.61	16.40	8.09
P. cuspidilimba	Currently unnamed	U−/−	85	18.50	3.53	27.14	11.43
P. dahlstedtii	Currently unnamed	U−/−	230	5.71	1.23	9.46	2.92
P. dolabella	*Tildenia*	No druses	...	...	...	...	...
P. dolabriformis	*Panicularia*	U−/+	55	11.54	1.81	15.52	7.59
P. emarginella	Currently unnamed	U−/−	108	10.97	1.87	17.93	7.59
P. fagerlindii	*Leptorhynchum*	U+/−	123	24.07	3.42	33.48	16.72
P. galioides	*Micropiper*	U−/−	198	18.70	2.38	26.28	18.70
P. glabella	*Micropiper*	$UV_{big}A_{sm}$−/−	320/386	9.15/5.88	1.48/.95	11.97/8.38	6.36/3.92
P. gracillima	*Tildenia*	U−/−	170	15.79	3.20	24.66	9.12
P. graveolens	*Panicularia*	U−/−	266	11.03	1.65	15.31	7.36
P. hoffmannii	Currently unnamed	U−/+	144	27.97	4.07	36.12	18.60
P. hylophila	*Micropiper*	U−/−	125	19.89	2.63	27.61	13.43
P. inaequalifolia	*Micropiper*	U−/−	88	11.92	1.64	15.44	8.09
P. incana	*Leptorhynchum*	UV_{under}+/−	252	8.70	1.28	11.85	5.19
P. lanceolatopeltata	*Oxyrhynchum*	U−/+ (large)	107	16.86	2.33	22.22	11.11
P. longespicata	*Leptorhynchum*	UV_{under}+/−	248	10.53	1.34	15.44	6.62
P. macrostachya	*Leptorhynchum*	U+/−	201	10.60	1.34	14.31	7.25
P. magnoliifolia	*Oxyrhynchum*	U−/−	202	7.95	1.13	11.11	5.19
P. marmorata	Currently unnamed	R−/−	164	12.42	1.66	16.23	8.18
P. maypurensis	Currently unnamed	$UV_{big}A_{sm}$−/−	247/415	6.99/3.12	.97/.89	8.86/5.43	5.55/1.51
P. metallica	*Peperomia*	$UV_{big}A_{sm}$−/−	345/155	18.87/8.78	2.71/8.78	25.45/13.20	13.30/5.68
P. pellucida	*Peperomia*	R−/−	245	15.48	2.20	21.12	10.47
P. pereskiifolia	Currently unnamed	U−/−	142	7.30	1.24	10.87	4.35
P. pernambucensis	*Oxyrhynchum*	U−/−	178	21.63	3.56	28.85	13.79
P. pitcairnensis	*Micropiper*	$UV_{big}A_{sm}$−/−	368/467	6.94/5.68	.99/.65	9.16/7.42	4.46/4.54
P. ppucuppucu	Currently unnamed	U−/−	224	16.79	2.24	23.21	10.71
P. prostrata	*Micropiper*	R−/−	415	14.75	2.22	22.16	7.61
P. reptilis	*Peperomia*	R−/−	172	19.90	2.96	27.09	13.03
P. resediflora	*Panicularia*	R−/+	102/208	9.69/12.28	1.29/2.06	13.04/17.73	7.25/6.95
P. rhombea	Currently unnamed	U−/−	328	16.73	2.42	21.74	11.23
P. rotundifolia	*Micropiper*	U−/−	546	7.90	1.28	11.95	4.63
P. serpens	*Leptorhynchum*	U+/−	444	7.80	1.39	10.90	3.86
P. sodiroi	Currently unnamed	U−/−	329	8.43	1.11	11.48	5.82
Peperomia sp.	*Micropiper*	U−/−	333	9.29	1.61	13.83	4.97
P. tetraphylla	Currently unnamed	U−/−	203	20.10	2.72	26.73	13.36
P. trifolia	Currently unnamed	U−/−	287	10.39	1.79	15.33	10.39
P. tristachya	Currently unnamed	$UV_{big}A_{sm}$−/−	298/300	7.68/4.88	1.22/.81	10.50/7.08	5.25/2.85
P. tuisana	*Micropiper*	R−/−	185	9.31	1.43	13.64	6.17
P. vinasiana	*Leptorhynchum*	UV_{under}+/−	198	8.98	1.62	13.05	5.43

Note. These numbers are representative for the specimens observed that include all of the identified macropatterns. Species are listed in alphabetical order. Infrageneric clades according to Samain et al. (2009).

of parsimony was performed with NONA, version 2.0, using the default settings of the program (Goloboff 1999). Evaluation of the tree was done by the bootstrap approach, conducting 1000 replicates and random addition searches with 10 iterations per cycle. Character states for the crystal macropatterns were compiled in a data set, and evolution of the patterns was investigated by plotting them on the phylogenetic tree using WINCLADA, version 1.00.08 (Nixon 2002), using the "unambiguous changes only" optimization option of the program (fig. 5).

Results

The general anatomy of *Peperomia* leaves—regardless of their size, shape, thickness, or coloration—consists of a typical single-layered adaxial epidermis and a single-layered

abaxial epidermis. Below the adaxial epidermis is a variable multilayered hypodermis consisting of large, highly vacuolate cells. At the base of this hypodermis is a typically, but not always, single-layered palisade parenchyma consisting of small cells packed with chloroplasts with little or no starch. Immediately below this layer and above the abaxial epidermis is the spongy parenchyma, containing starch, in which the vascular bundles (veins) are found immediately under the palisade parenchyma. Even though the size, shape, and surface topography of the leaves vary widely, this anatomical arrangement seems to be consistent (Haberlandt 1904; Schürhoff 1908; Franceschi and Horner 1980; F. Foulon, M.-S. Samain, and P. Goetghebeur, unpublished data; fig. 1).

Forty-four of the 45 species examined display crystals in the palisade parenchyma and sometimes in the spongy parenchyma (fig. 1); one species (*Peperomia dolabella*) did not contain any druses. These different and distinct crystal arrangements allowed us to categorize their presence in one or two parenchyma regions (palisade parenchyma only or palisade and spongy parenchymas) into crystal macropatterns (sensu Lersten and Horner 2000). The designations used for these patterns are divided into two main categories: druses throughout the palisade parenchyma (uniform [U]) and druses in the palisade parenchyma limited to only the palisade cells over the subtending veins (reticulate [R]). Variations of these two main patterns include the absence or presence of crystals in the spongy parenchyma (U−/−, R−/−, or U +/−, U−/+, R+/−, R−/+). If raphide crystal bundles occur in the spongy parenchyma for either of the two major patterns, the designation is U or R (+/−); if, on the other hand, prisms occur in the spongy parenchyma, the designation is U or R (−/+). We have never observed the presence of both raphides and prisms in the spongy parenchyma at the same time or in the same species. In some species having the U pattern, druses over the veins may be larger (V_{big}) than druses over the areole regions (A_{sm}), thus, the designation $UV_{big}A_{sm}(-/-)$. One other variation of U is where the raphide bundles only exist under the veins ($U+V_{under}/-$).

Generally, druses occur singly only in the typically single-layered palisade parenchyma. Sometimes, there was more than one palisade parenchyma layer, which placed the druses in different planes of focus. Two most conspicuous leaf macropatterns exist among 44 species having crystals. In 81.8% (36) of the species, all or almost all parenchyma cells from margin to margin typically contain one druse each. This macropattern is termed "uniform" (table 1). In the remaining 18.2% (8) of the species, the druses are located in the palisade parenchyma only above the veins. This macropattern is termed "reticulate" (table 1). Each of these two major macropatterns is further subdivided depending on the absence of both or the presence of either raphide bundles or prisms in the spongy parenchyma or differences in the sizes between druses over the veins and over the areoles (table 1).

The most prominent macropattern is the absence of both raphide bundles (U−/_, where the underscore indicates the

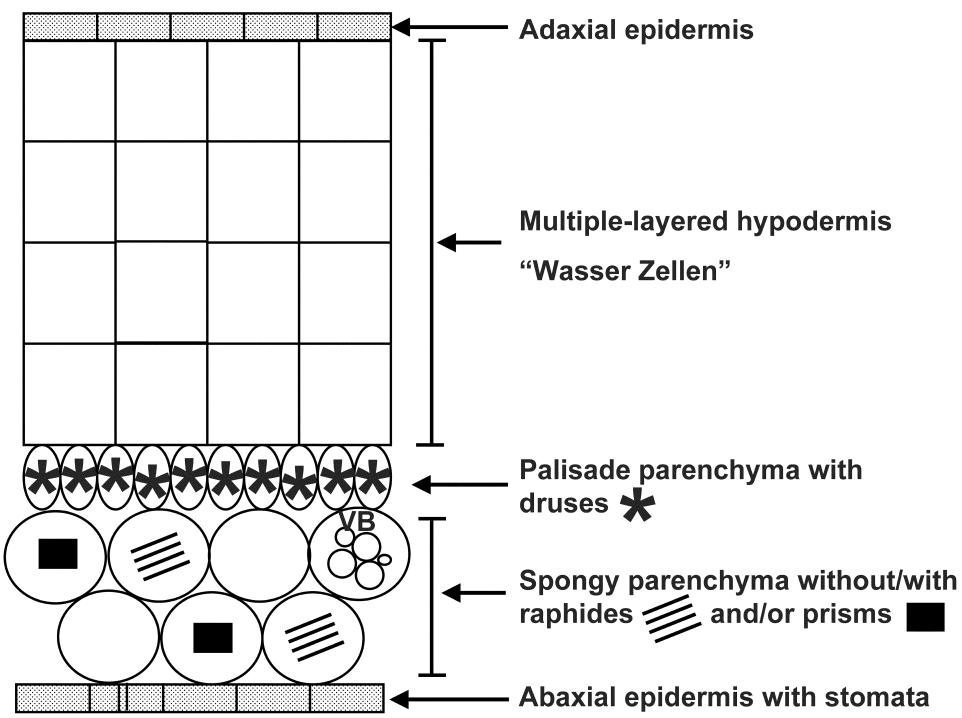

Fig. 1 Drawing of cross section of idealized mature *Peperomia* leaf showing two uniseriate epidermal layers, a multilayered hypodermis, a palisade parenchyma layer with either all cells each containing a druse or only selected cells containing individual druses, and a spongy parenchyma with vascular bundles making up the venation system. In some species, the spongy parenchyma may be devoid of crystals altogether, it may contain raphide idioblasts, or it may contain prisms but not both types of crystals. The presence of one or more types of crystals in different locations creates a distinct crystal macropattern.

absence of raphide bundles) and prisms (U_/−, where the underscore indicates the absence of prisms) in the spongy parenchyma (= U−/−; fig. 2*A*–2*D*). This macropattern occurs in 50.0% (22) of the species. The modifications of this most common macropattern are the following: U with larger druses over the veins and smaller druses over the areole regions (= $UV_{big}A_{sm}$−/−; fig. 2*E*–2*G*), 11.4% (five species); U with raphide bundles throughout the spongy parenchyma (= U+/−; fig. 2*H*–2*J*), 6.8% (three species); U with prisms throughout the spongy parenchyma (= U−/+; fig. 3*A*, 3*B*, where arrowheads identify prisms), 6.8% (three species); and U with raphide bundles only under the veins (= $U+V_{under}$/−; fig. 3*C*–3*F*), 6.8% (three species; see table 1).

The most common macropattern of U−/− occurs throughout the phylogenetic tree (see fig. 5) and is the only macropattern found in most but not all of the clades (table 1). Species displaying this most common pattern are *P. andina* var. *pseudoperuviana*, *P. bicolor*, *P. blanda*, *P. clusiifolia*, *P. cuspidilimba*, *P. dahlstedtii*, *P. emarginella*, *P. galioides*, *P. gracillima*, *P. graveolens*, *P. hylophila*, *P. inaequalifolia*, *P. magnoliifolia*, *P. pereskiifolia*, *P. pernambucensis*, *P. ppucuppucu*, *P. rhombea*, *P. rotundifolia*, *P. sodiroi*, *Peperomia* sp., *P. trifolia*, and *P. tetraphylla*.

Three variations of the U macropattern include U+/− (*P. fagerlindii*, *P. macrostachya*, and *P. serpens*), $U+V_{under}$/− (*P. incana*, *P. longespicata*, and *P. vinasiana*), and U−/+ (*P. dolabriformis*, *P. hoffmannii*, and *P. lanceolatopeltata*). The first two variations are restricted to a major clade represented by six species (see fig. 5 for explanation). Species displaying $U+V_{under}$/− seem to have a different leaf anatomy that allows for this specific crystal arrangement. This third variation occurs in three species that are not phylogenetically related.

The final U macropattern variation, consisting of five species, displays the macropattern $UV_{big}A_{sm}$−/− and is found scattered in different clades represented by *P. glabella*, *P. maypurensis*, *P. metallica*, *P. pitcairnensis*, and *P. tristachya*. These species show different sizes (diameters) of druses, with larger ones over the veins and smaller ones over the areoles between the venation (table 1).

The R macropattern consists of two variations. The more common macropattern is defined by druses over the veins and no crystals in the spongy parenchyma (R−/−; fig. 3*G*, 3*H*), 16% (seven species), and is represented by *P. cotyledon*, *P. argyreia*, *P. marmorata*, *P. pellucida*, *P. prostrata*, *P. reptilis*, and *P. tuisana*, belonging to different clades (fig. 5). The other variation is characterized by druses over the veins and prisms in the spongy parenchyma (R−/+; fig. 3*I*, 3*J*), 2.2% (one species; *P. resediflora*).

Peperomia maypurensis and *P. metallica* (both $UV_{big}A_{sm}$−/−) and *P. cotyledon* (R−/−) have the distinction of variably displaying no druses or few (not shown) to many (fig. 3*K*, 3*L*) druses in the areole regions of different leaves. More collections of each of these species will need to be studied to understand this variability.

The sizes of the druses were visibly different between the species as shown in micrographs taken at the same magnification (range in mean diameter from smallest druses in *P. andina* var. *pseudoperuviana* of 4.27 μm to the largest druses in *P. hoffmannii* of 27.97 μm; table 1). The mean difference in volume between sizes of druses of these two species is a factor of 281. No length measurements were made for the prisms and raphide bundles.

Several species with the U−/− macropattern visibly displayed a concentration of druses over the veins due to leaf shape, or the region over the veins was depressed, making the druses appear absent or out of focus. Four species showed this former condition of visual concentration of druses over the veins, thus making the venation appear more prominent: *P. bicolor* (fig. 4*A*), *P. sodiroi* (fig. 4*B*), *P. glabella* (fig. 4*C*), and *P. rotundifolia* (not shown). The latter of the two conditions shows a depressed druse layer over the veins in *P. clusiifolia* (fig. 4*D*, 4*E*). In addition, *P. pernambucensis* (fig. 4*F*) displayed prominent thick and highly polarized veins in its cleared leaves. These prominent veins are more conspicuous than in any of the other 44 species studied. Finally, *P. ppucuppucu* displayed a highly packed layer of druses that extended all the way to the leaf margin (fig. 4*G*). All of these conditions add to the complexity of the variations seen in *Peperomia* crystal macropatterns.

Discussion

Evolution of Crystal Macropatterns in Peperomia

This is the first study that directly compares leaf calcium oxalate crystal macropatterns with complementary molecular data. Our study has focused on the genus *Peperomia* because it is one of the largest angiosperm genera, displaying an extreme diversity of leaf texture, shape, succulence, lamina size and thickness, coloration, venation, and crystals. In addition, a robust and well-supported backbone phylogeny based on molecular and morphological data is available for all studies within this genus (Wanke et al. 2006; Samain et al. 2009). Hence, *Peperomia* represents a challenging opportunity to investigate leaf calcium oxalate crystal macropattern variation and evolution in a monophyletic yet highly diverse genus. To date, our findings in *Peperomia* cannot be discussed in the wider framework of the family Piperaceae because leaf anatomy and the presence of crystals in the other genera have been studied very little or not at all. In addition, leaf crystals in the few investigated species of the sister genus *Piper* (Souza et al. 2004) seem to be very different from those in *Peperomia*. Brief observations (H. T. Horner, unpublished data) of cleared leaf punches from two available species of *Piper* showed that *Piper nigrum* displayed both large and smaller crystal sand cells in the leaf lamina, whereas *Piper peltatum* contained very small individual crystals throughout the leaf lamina. These two types of crystals were not observed in any of the *Peperomia* species in this study. Therefore, without a more extensive survey of the genus *Piper* or the other genera allied to *Peperomia*, it is impossible to draw any conclusions about crystal macropattern evolution in the family Piperaceae.

Leaf anatomy of *Peperomia* has been extensively studied for some species (Schürhoff 1908; Johnson 1914; Yuncker and Gray 1934; Skottsberg 1947; Metcalfe and Chalk 1950; Murty 1960; Datta and Dasgupta 1977; Kaul 1977; Franceschi and Horner 1980; Christensen-Dean and Moore 1993; Takemori et al. 2003). Although most of these studies included

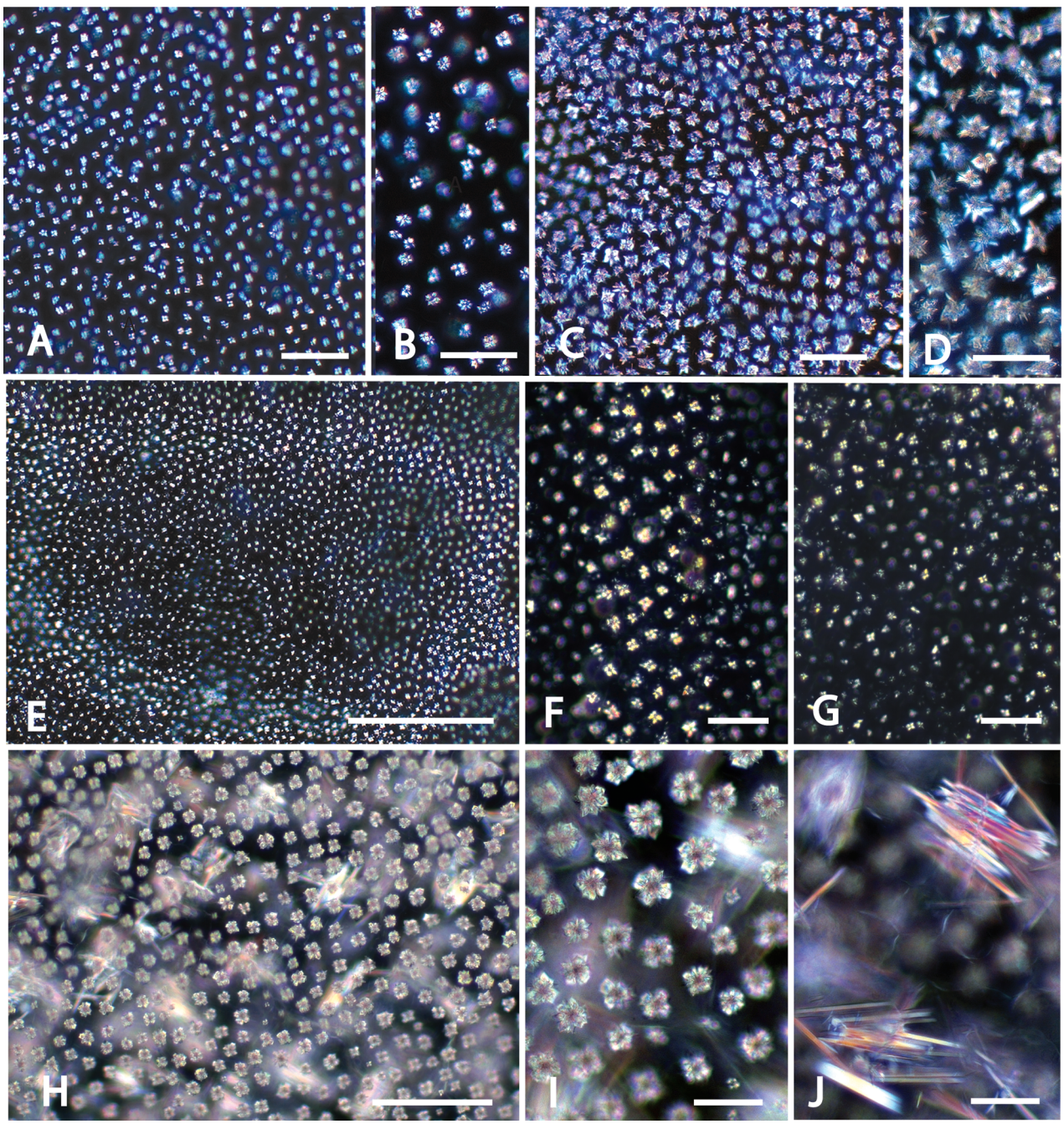

Fig. 2 Cleared leaves of *Peperomia* species observed between crossed polarizers to observe variations in crystal macropatterns (U) at low and higher magnifications. Images depict druses in palisade parenchyma and prisms or raphides in spongy parenchyma. *A*, *Peperomia. bicolor* palisade parenchyma druses (U−/−). *B*, *Peperomia bicolor* palisade parenchyma druses at higher magnification (U−/−). *C*, *Peperomia galioides* palisade parenchyma druses (U−/+). *D*, *Peperomia galioides* palisade parenchyma druses at higher magnification with underlying prisms in spongy parenchyma (U−/+). *E*, *Peperomia tristachya* palisade parenchyma druses showing large druses (over veins) and smaller druses (areole region; $UV_{big}A_{sm}$−/−). *F*, *Peperomia tristachya* druses over vein at higher magnification ($UV_{big}A_{sm}$−/−). *G*, *Peperomia tristachya* druses in areole region ($UV_{big}A_{sm}$−/−). *H*, *Peperomia fagerlindii* palisade parenchyma druses with underlying spongy parenchyma raphide crystal bundles (U+/−). *I*, *Peperomia fagerlindii* palisade parenchyma druses at higher magnification, each displaying a prominent central core (U+/−). *J*, *Peperomia fagerlindii* spongy parenchyma bundles showing some individual raphides (U+/−). Bars = 100 μm in *A*, *C*; 50 μm in *B*, *D*, *F*, *G*, *I*, *J*; 200 μm in *E*, *H*.

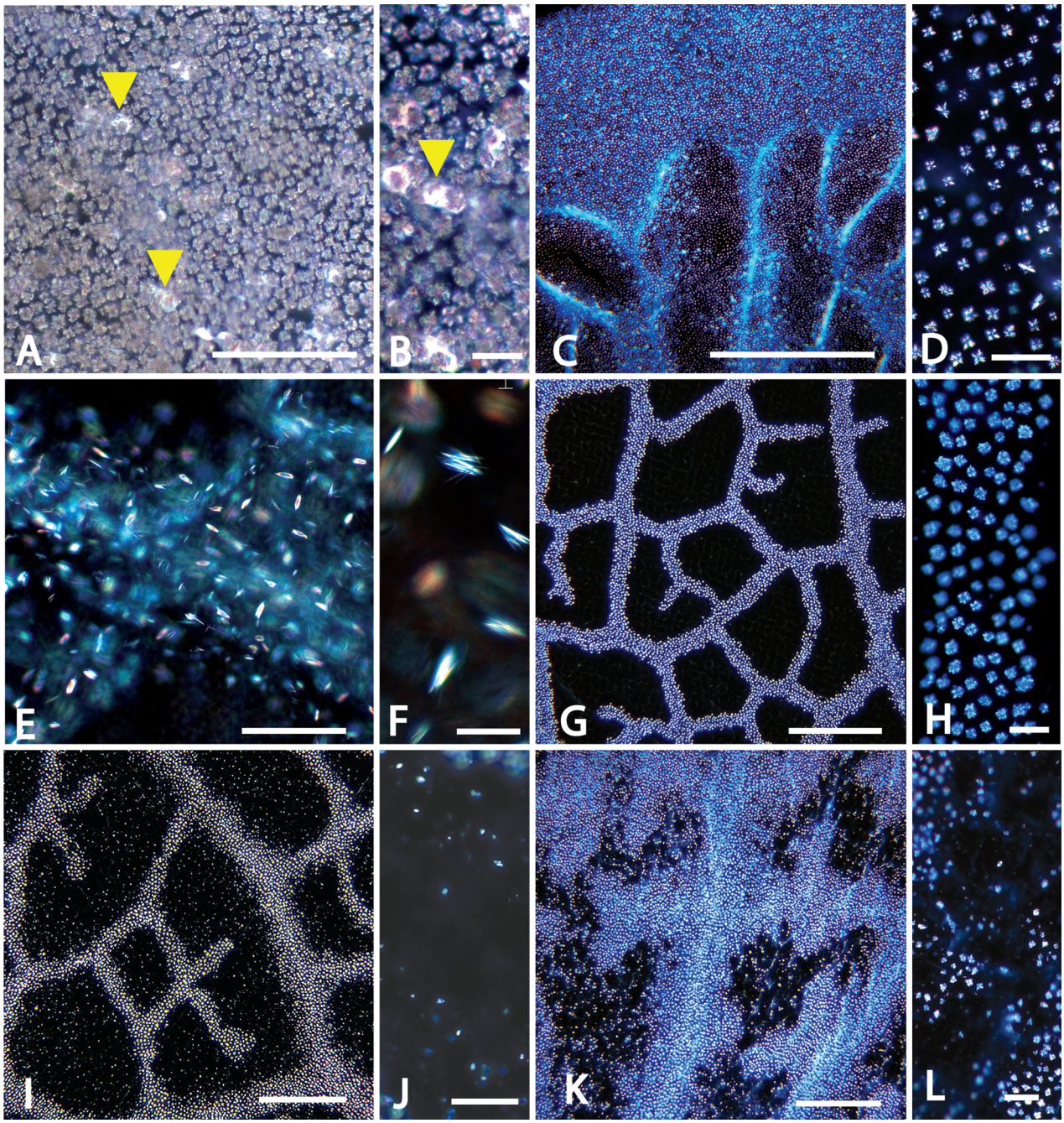

Fig. 3 Cleared leaves of *Peperomia* species observed between crossed polarizers to observe variations in major crystal macropatterns (uniform [U], reticulate [R]) and their modifications at low and higher magnifications. Images depict druses in palisade parenchyma and raphides in spongy parenchyma. *A*, *Peperomia lanceolatopeltata* (U−/+), densely packed palisade parenchyma druses with prisms (arrowheads) in spongy parenchyma. *B*, *Peperomia lanceolatopeltata*, showing *A* at higher magnification; the arrowhead identifies a prism. *C*, *Peperomia vinasiana* ($U+V_{under}/-$), showing uniform druses around margin lamina and $U+V_{under}/-$ in inner lamina. *D*, *Peperomia vinasiana* druses around margin lamina. *E*, *P. vinasiana* in inner lamina with druses over veins and raphides under veins. *F*, *Peperomia vinasiana*, showing raphides under veins at higher magnification. *G*, *Peperomia marmorata* (R−/−), with druses restricted to lamina regions only over veins. *H*, *Peperomia marmorata*, showing portion of vein-associated druses at higher magnification. *I*, *Peperomia resediflora* (R−/+), showing druses over veins and prisms in areole regions. *J*, *Peperomia resediflora*, showing areole prisms at higher magnification. *K*, *Peperomia cotyledon* (R−/−), showing a spread-out array of druses that extend beyond veins with some indiscriminately occurring in some areole regions. *L*, *Peperomia cotyledon*, with areole druses at higher magnification. Bars = 100 μm in *A*; 50 μm in *B*, *D*, *F*, *H*, *J*, *L*; 1000 μm in *C*; 500 μm in *E*, *G*, *I*, *K*.

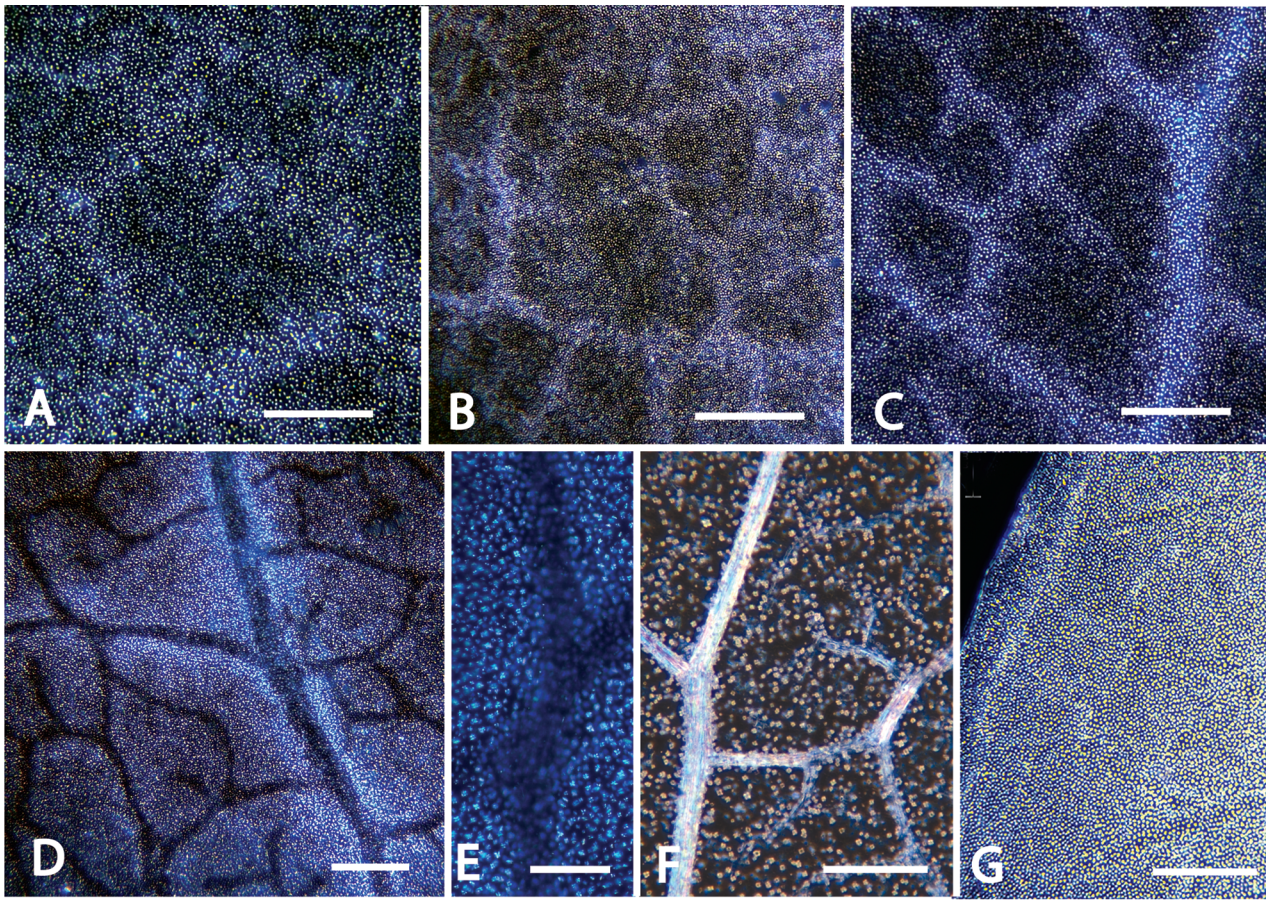

Fig. 4 Cleared leaves of *Peperomia* species observed between crossed polarizers to show variations in crystal macropatterns (uniform [U]) at low and higher magnifications. Images depict druses in palisade parenchyma. *A*, *Peperomia bicolor* (U−/−), showing an enhancement of venation due to concentration of druses over veins. *B*, *Peperomia sodiroi* (U−/−), showing a greater enhancement of venation than in *A* due to concentration of druse over veins. *C*, *Peperomia glabella* (U−/−), showing an even greater enhancement of venation than in *B* due to concentration of druses over veins. *D*, *Peperomia clusiifolia* (U−/−), with depressed lamina over veins. *E*, *Peperomia clusiifolia*, showing a portion of venation and surrounding lamina druses. *F*, *Peperomia pernambucensis* (U−/−), with very distinct venation as compared with all other species in this study. *G*, *Peperomia ppucuppucu* (U−/−), with densely packed lamina druses that extend to near the leaf margin. Bars = 500 μm in *A–D*, *F*, *G*; 100 μm in *E*.

only a few species, F. Foulon, M.-S. Samain, and P. Goetghebeur (unpublished data) sampled more widely within the genus; however, the distribution of crystals and their shapes has never been investigated for a representative sampling of the genus until this study.

Haberlandt (1904), Schürhoff (1908), and Franceschi and Horner (1980) noted that *Peperomia* druses occurred specifically in the photosynthetic palisade parenchyma and that other types of crystals sometimes occurred in the spongy parenchyma (Franceschi and Horner 1980). This latter study indicated that isolated druses from several species had different distinct shapes of the crystal facets that suggested druse microstructure may be species specific and potentially could be used as a taxonomic character. This particular aspect is yet to be pursued in depth.

With one exception among the 45 species (*Peperomia dolabella* has no druses in the specimens studied), 36 of the 44 species display druses in all or almost all of their palisade parenchyma cells (U), and no other crystal shapes are present. The remaining eight species display palisade parenchyma druses above the veins only (R). These two major crystal macropatterns together confirm that the genus *Peperomia* is consistent in having only druses in its palisade parenchyma, a unifying crystal character for the genus. The other five variations of these macropatterns provide a foundation for assessing them in relation to the phylogeny of the genus.

Following the placement of the seven patterns on the phylogenetic tree, several evolutionary trends become apparent (fig. 5). Crystal macropattern evolution in *Peperomia* is generally characterized by an increasing complexity of the distribution of druses, raphides, and prisms. However, this trend is partially interrupted by reversals. The occurrence of palisade parenchyma druses distributed uniformly throughout the leaf lamina (U−/−) is considered the ancestral state in the genus, and reversals to this particular state were not observed. This state shifts to more complex crystal macropatterns with either the presence of raphides or prisms in addition to the druses and to the occurrence of druses above the venation

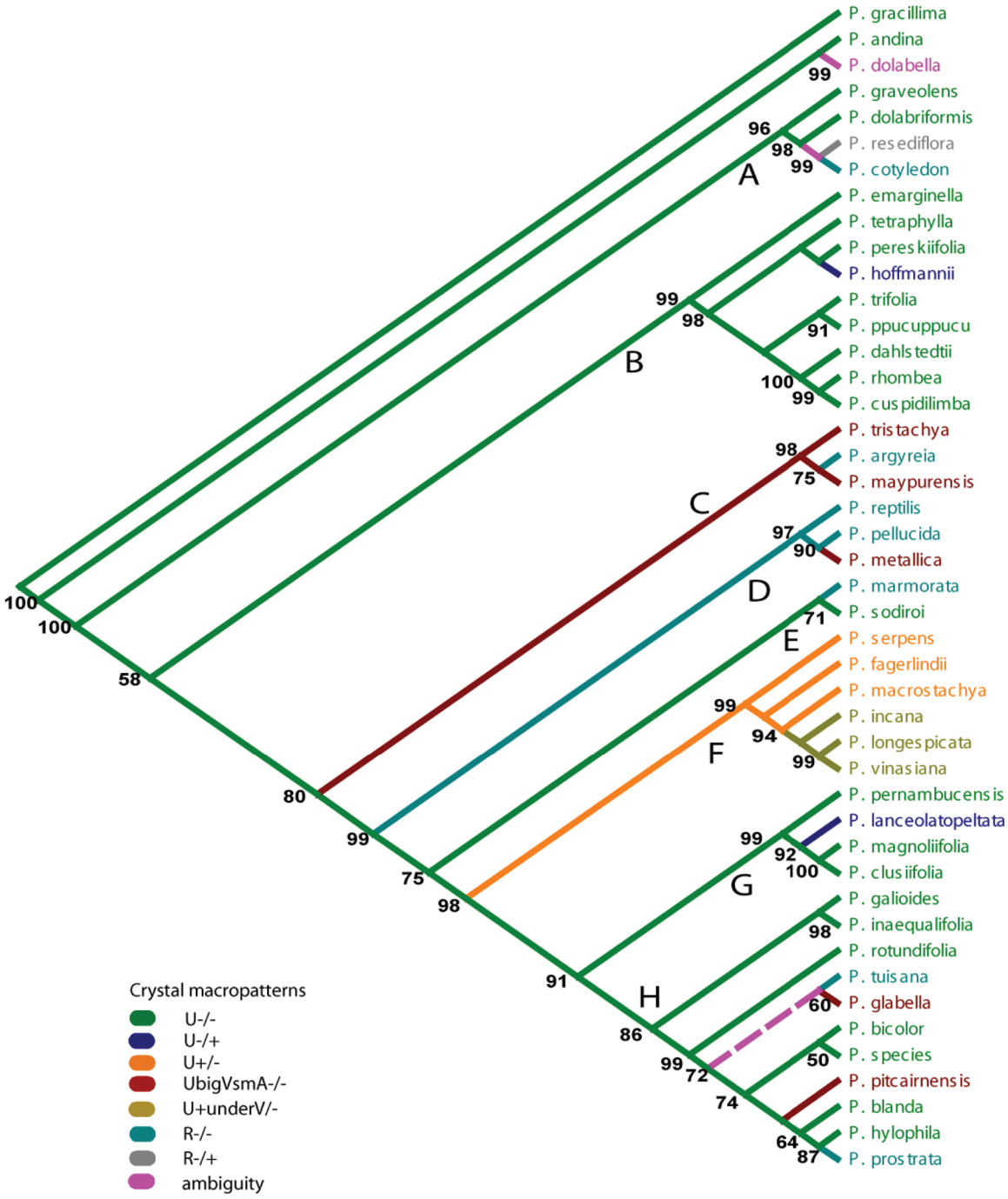

Fig. 5 Character optimization, based on a parsimony approach using the chloroplast *trnK/matK* region (Wanke et al. 2006) in *Peperomia*, showing the seven crystal macropatterns. Forty-five species were observed in this study, and one (*Peperomia dolabella*) had no druses. The subgenus *Tildenia* (see table 1) is used as an outgroup for the analysis. The phylogenetic tree recovered eight clades, to which letters are assigned: *A* = subgenus *Panicularia*; *B* = *Pleurocarpidium* + a currently unnamed subgenus; *C* = currently unnamed subgenus; *D* = subgenus *Peperomia*; *E* = currently unnamed subgenus; *F* = subgenus *Leptorhynchum*; *G* = subgenus *Oxyrhynchum*; *H* = subgenus *Micropiper*. Bootstrap support values are indicated above the branches. The macropatterns are U−/− (uniform druses in palisade parenchyma, no crystals in spongy parenchyma), U+/− (uniform druses in palisade and raphide crystal idioblasts in spongy parenchyma), U+V_{under}/− (uniform druses in palisade parenchyma and raphide crystal idioblasts only under veins), U−/+ (uniform druses in palisade and prisms in spongy parenchyma), $UV_{big}A_{sm}$−/− (large druses in palisade over veins and smaller druses in palisade over areoles, no crystals in spongy parenchyma), R−/− (druses in palisade over the veins, no druses over areoles, and no crystals in spongy parenchyma), and R−/+ (druses in palisade over veins and prisms in spongy parenchyma).

only. Hence, it can be hypothesized that in species characterized by the ancestral state (U−/−), crystal formation is basically involved in calcium regulation/sequestration (Volk et al. 2002; Nakata 2003). The presence of larger druses over the veins and smaller druses over the areoles ($UV_{big}A_{sm}$−/−), as well as of druses over the veins only (R), might indicate that the druses are not merely a waste or storage product (sequestration) but potentially protect or in some other way interact with the vascular tissues (Volk et al. 2002; Nakata 2003). Finally, the occurrence of raphides and prisms also seems to occur in more derived species (fig. 5).

The variation of the U macropattern where druses are larger over the veins and smaller in the areole regions ($UV_{big}A_{sm}$−/−) is represented by five species (table 1). Measurable differences in the mean diameter of the druses in both regions indicate a tantalizing possibility that this third macropattern may be an intermediate condition between U and R. This is partially confirmed by the optimization of the crystal macropattern on the phylogenetic tree (fig. 5), where it is shown in clade C that the $UV_{big}A_{sm}$−/− pattern originates from the U−/− pattern and further evolves to the R−/− pattern. However, other species displaying the $UV_{big}A_{sm}$−/− macropattern are found individually in distant clades C, D, and H (fig. 5). Three of the species (table 1), on the other hand, are associated with species displaying R−/− in adjacent clades C and D (fig. 5). The other two species occur among species displaying U−/− in the H clade (fig. 5).

Species displaying R−/−, with three exceptions, are located together (fig. 5) in clades C, D, and E. As just mentioned, the other species in these three clades display $UV_{big}A_{sm}$−/−, a potentially intermediate macropattern.

Another macropattern that is clade associated is U+/−, where raphide crystal bundles either occur uniformly in the spongy parenchyma or are primarily under the veins ($U+V_{under}$/−). Six species are represented by this macropattern and are grouped together in clade F (fig. 5). The presence of raphides is a clear synapomorphy for this clade. According to previous authors (Volk et al. 2002; Nakata 2003), raphides may have a dual function of calcium regulation and plant defense, whereas druses are involved only in calcium regulation/sequestration. The fact that raphides in *Peperomia* occur only in more derived clades supports the notion that crystals in plants are involved secondarily in other strategies than merely calcium regulation, including plant defense and protection. A similar trend has also been observed in *Prunus* (Rosaceae), where species of the most evolved subgenera are characterized by the presence of druses over the venation only (Lersten and Horner 2000).

Another explanation of the variations in the crystal macropatterns and their functional significance goes back to two earlier studies, those of Schürhoff (1908) and Haberlandt (1904), and later to those of Horner (1976), Franceschi and Horner (1980), Kuo-Huang et al. (2007), and Horner (2008). All of these studies have suggested that druses in the palisade parenchyma could serve as light-gathering and light-reflecting systems for photosynthesis in the small-celled palisade parenchyma. Kuo-Huang et al. (2007) carried out experiments by growing *P. glabella* under different light intensities and then compared druse diameters and their positions within the palisade cells under each intensity condition. Their preliminary results support the idea that druses play a functional role in the control of light gathering and dissemination to the chloroplasts. However, additional studies still need to be carried out to confirm this tantalizing possibility and to demonstrate that crystals do play one of possibly several important roles in the physiology of plant systems.

What the contributing role(s) of the raphide bundles and prisms is/are in the spongy parenchyma is unknown. The crystal macropatterns identified in this study could be considered from this point of view, particularly with respect to the environments the different species inhabit. Could the druses specifically be serving as light-gathering and light-reflecting systems for the chloroplasts surrounding them? This is a tantalizing idea that requires further work to compare multiple factors such as leaf anatomy and morphology, phylogeny, and crystal development using species with specific macropatterns grown under different light regimes. Such studies could provide significant information about how some plants, in general, are able to adapt successfully to their different environments if crystals are involved.

Systematic Value of the Leaf Crystal Macropatterns in Peperomia

A number of unrelated previous studies support the idea that the formation of calcium oxalate crystals, including their individual shapes, hydration forms, and locations within the plant body, is species specific and under genetic control (Kausch and Horner 1982; Zindler-Frank 1987; Horner and Wagner 1995; Lersten and Horner 2000). Hufford (1997) carried out a survey of Hydrangeaceae in which he used a cladogram to define three crystal groups in different clades within the family. His results indicated that crystal type and distribution were significant for the systematics of this family. Lersten and Horner (2000) identified six leaf crystal macropatterns in 131 species of the genus *Prunus* (Rosaceae) and showed that there were general trends of macropatterns among the five subgenera. These results were, at that time, not supported by any molecular data. However, Lee and Wen (2001) carried out a slightly later molecular systematic study, independent of that of Lersten and Horner (2000), that supported the five subgenera they used for their crystal classification. Cervantes-Martinez et al. (2005) showed similar trends in related subgroups within the Glycine complex. These complementary studies provided the supporting evidence that crystal macropatterns potentially could be a useful taxonomic character at the genus and subgenus level or even below.

If these previous studies are correct, then the crystal macropatterns should be at least partially congruent with the phylogeny of the genus *Peperomia*. The question to be asked for *Peperomia* is whether the crystal macropatterns identified in this study fit the phylogenetic hypotheses based on molecular data. One of the major problems in the study of the species-rich genus *Peperomia* is the search for phylogenetically informative characters, both qualitative and quantitative. The lack of useful characters complicates the delimitation of monophyletic infrageneric groups. As a consequence of the parallel evolution of many morphological characters, these infrageneric clades generally are characterized by a unique combination of characters rather than by a single synapomorphy (Samain

et al. 2009). This also counts for the crystal macropatterns, although they are a potential synapomorphy for some clades. Especially interesting is the occurrence of raphides (U+/− and $U+V_{under}/-$) in clade F (fig. 5), a morphologically easily distinguishable clade consisting of epiphytic species with coriaceous leaves; relatively large, elongated cylindrical fruits; and sclerenchyma above the vascular bundles. Two currently unnamed clades in this analysis, each represented by three species, are characterized by the potentially developmentally closely related patterns $UV_{big}A_{sm}-/-$ and R−/− (clades C and D; fig. 5). Although both patterns also sporadically occur in other clades, their presence in these neighboring clades is conspicuous. Both clades consist of epiphytic or terrestrial species, but species of the first clade (currently unnamed) all have peltate leaves, whereas the second group (*Peperomia*) contains delicate plants of which at least one is an annual species, an unusual life-form in the genus.

The other clades are not characterized by any specific crystal macropattern. Hence, crystal occurrence and distribution are not highly congruent with phylogenetic hypotheses within *Peperomia*. Nevertheless, these data form an important contribution to the current knowledge of *Peperomia* and are an additional character useful for a new infrageneric classification of the genus.

Conclusion

Evolution of leaf crystal macropattern in the genus *Peperomia* is characterized by an increasing complexity of the distribution of different crystals types, with few reversals. The occurrence of raphides and prisms and the presence of druses over the veins characterize only more evolved clades. This supports the hypothesis that crystal formation in plants does not exclude but seems to be secondarily involved in strategies other than merely calcium regulation, including plant defense, protection, and photosynthetic efficiency. Although crystal macropatterns are not highly congruent with previous studies based on their macropattern and molecular data, they count for nearly all, up to now, observed characters in the genus, resulting in the fact that infrageneric clades are characterized by a unique combination of characters rather than by a single synapomorphic character. The results in *Peperomia* confirm previous studies in several unrelated plant groups where a systematic correlation of the leaf crystals has been demonstrated. However, to get a complete insight and overview of the systematic significance of crystals in angiosperms, a larger survey of their presence, function, and distribution is required.

Acknowledgments

We thank the following units for their support: the Microscopy and NanoImaging Facility, Iowa State University (ISU), for use of their laboratory, microscope, and imaging instrumentation; the Department of Genetics, Development, and Cell Biology (ISU); the Botanical Gardens at Ghent and Dresden, as well as the Research Foundation-Flanders (FWO, G.0172.07); and the German Science Foundation (DFG, NE 681/5-1) for financial support. In addition, we thank the following individuals: Steve Mahoney (EEOB) and Arlen Patrick (Horticulture), greenhouse managers; Deborah Lewis, curator of the Ada Hayden Herbarium (ISC); Katherine Swanson, an undergraduate honors student at ISU; Dr. Paul Goetghebeur and Dr. Christoph Neinhuis, directors of the botanical gardens of Ghent and Dresden; and Helga Ochoterena (UNAM, Mexico) and Dr. Nels R. Lersten (ISU) for critically annotating our manuscript, as well as the two anonymous IJPS reviewers for their constructive comments.

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